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## DNA Purification

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## **WHAT CRITERIA COULD YOU CONSIDER WHEN SELECTING A PURIFICATION STRATEGY?**

### **How Much Purity Does Your Application Require?**

What contaminants will affect your immediate and downstream application(s)? As discussed below and in Chapter 1, “Planning for Success in the Laboratory,” time and money can be saved by determining which contaminants need not be removed. For example, some PCR applications might not require extensively purified DNA. Cells can be lysed, diluted, and amplified without any further steps. Another reason to accurately determine purity requirements is that yields tend to decrease as purity requirements increase.

### **How Much Nucleic Acid Can Be Produced from a Given Amount of Starting Material?**

While it is feasible to mathematically calculate the total amount of nucleic acid in a given sample, and values are provided in the research literature (Sambrook et al., 1989; Studier and Moffat, 1986; Bolivar et al., 1977; Kahn et al., 1979; Stoker et al., 1982), the yields from commercial purification products and noncommercial purification strategies are usually significantly less than these maxima, sometimes less than 50%. Since recoveries will vary with sample origin, consider making your plans based on yields published for samples similar if not identical to your own.

### **Do You Require High Molecular Weight Material?**

The average size of genomic DNA prepared will vary between commercial products and between published procedures.

### **How Important Is Speed to Your Situation?**

Some purification protocols are very fast and allow isolation of nucleic acids within 30 minutes, but speed usually comes at the price of reduced yield and/or purity, especially when working with complex samples.

### **How Important Is Cost?**

Reagents obviously figure into the cost of a procedure, but the labor required to produce and apply the reagents of purification should also be considered.

### **How Important Is Reproducibility (Robustness) of the Procedure?**

Some methods will not give consistent quality and quantity. When planning long-term or high-throughput extractions, validate your methods for consistency and robustness.

### **What Interferes with Nucleic Acid Purification?**

#### *Nuclease*

One of the major concerns of nucleic acid purification is the ubiquity of nucleases. The minute a cell dies, the isolation of DNA turns into a race against internal degradation. Samples must be lysed fast and completely and lysis buffers must inactivate nucleases to prevent nuclease degradation.

Most lysis buffers contain protein-denaturing and enzyme-inhibiting components. DNases are much easier to inactivate than RNases, but care should be taken not to reintroduce them during or after purification. All materials should be autoclaved or baked four hours at 300°F to inactivate DNases and RNases, or you should use disposable materials. Use only enzymes and materials guaranteed to be free of contaminating nucleases. Where appropriate, work on ice or in the cold to slow down potential nuclease activity.

Smears and lack of signal, or smeared signal alone, and failure to amplify by PCR are indicative of nuclease contamination. The presence of nuclease can be verified by incubating a small aliquot of your sample at 37°C for a few hours or overnight, followed by evaluation by electrophoresis or hybridization. If nuclease contamination is minor, consider repurifying the sample with a procedure that removes protein.

#### *Shearing*

Large DNA molecules (genomic DNA, bacterial artificial chromosomes, yeast artificial chromosomes) can be easily sheared during purification. Avoid vortexing, repeated pipetting (especially through low-volume pipette tips), and any other form of mechanical stress when the isolate is destined for applications that require high molecular weight DNA.

### *Chemical Contaminants*

Materials that interfere with nucleic acid isolation or downstream applications involving the purified DNA can originate from the sample. Plants, molds, and fungi can present a challenge because of their rigid cell wall and the presence of polyphenolic components, which can react irreversibly with nucleic acids to create an unusable final product.

The reagents of a DNA purification method can also contribute contaminants to the isolated DNA. Reagents that lyse and solubilize samples, such as guanidinium isothiocyanate, can inhibit some enzymes when present in trace amounts. Ethanol precipitation of the DNA and subsequent ethanol washes eliminate such a contaminant. Phenol can also be problematic. If you experience problems with DNA purified by a phenol-based strategy, apply chloroform to extract away the phenol. Phenol oxidation products may also damage nucleic acids; hence re-distilled phenol is recommended for purification procedures.

A mixture of chloroform and phenol is often employed to maximize the yield of isolated DNA; the chloroform reduces the amount of the DNA-containing aqueous layer at the phenol interphase. Similar to phenol, residual chloroform can be problematic, and should be removed by thorough drying. Drying is also employed to remove residual ethanol. Overdried DNA can be difficult to dissolve, so drying should be stopped shortly after the liquid can no longer be observed. Detailed procedures for the above extraction, precipitation and washing steps can be found in Sambrook, Fritsch, and Maniatis (1989) and Ausubel et al. (1998).

Ammonium ions inhibit T4 polynucleotide kinase, and chloride can poison translation reactions (Ausubel et al., 1998). The common electrophoresis buffer, TBE (Tris, borate, EDTA) can inhibit enzymes (Ausubel et al., 1998) and interfere with transformation due to the increased salt concentration (Woods, 1994). Phosphate buffers may also inhibit some enzymes, namely T4 Polynucleotide kinase (Sambrook et al., 1989), alkaline phosphatase (Fernley, 1971), Taq DNA polymerase (Johnson et al., 1995), and Poly A polymerase from *E. coli* (Sippel, 1973). Agarose can also be a problem but some enzyme activity can be recovered by adding BSA to 500  $\mu\text{g/ml}$  final concentration (Ausubel et al., 1998). EDTA can protect against nuclease and heavy metal damage, but could interfere with a downstream application.

The anticoagulant heparin can contaminate nucleic acids isolated from blood, and should be avoided if possible (Grimberg et al., 1989). Taq DNA polymerase is inhibited by heparin, which

can be resolved by the addition of heparinase (Farnert et al., 1999). Heparin also interacts with chromatin leading to release of denatured/nicked DNA molecules (Strzelecka, Spitkovsky, and Paponov, 1983). Narayanan (1996) reviews the effects of anticoagulants.

### **What Practices Will Maximize the Quality of DNA Purification?**

The success of DNA purification is dependent on the initial quality of the sample and its preparation. It would be nice to have a simple, straightforward formula that applies to all samples, but some specimens have inherent limitations. The list below will help guide your selection and provide remedies to nonideal situations:

1. Ideally start with fresh sample. Old and necrotic samples complicate purification. In the case of plasmid preparations, cell death sets in after active growth has ceased, which can produce an increase in unwanted by-products such as endotoxins that interfere with purification or downstream application.

The best growth phase of bacterial cultures for plasmid preparations may be strain dependent. During the log phase of bacterial culture, actively replicating plasmids are present that are “nicked” during replication rather than being supercoiled. Still some researchers prefer mid to late log phase due to the high ratio of DNA to protein and low numbers of dead cells. Others only work with plasmids that have grown just out of log phase to avoid co-purification of nicked plasmid.

If old samples can't be avoided, scaling up the purification can compensate for losses due to degradation. PCR or dot blotting is strongly recommended to document the integrity of the DNA.

2. Process your sample as quickly as possible. There are few exceptions to this rule, one being virus purification. When samples can't be immediately purified, snap freeze the intact sample in liquid nitrogen or hexane on dry ice (Franken and Luyten, 1976; Narang and Seawright, 1990) or store the lysed extract at  $-80^{\circ}\text{C}$ . Commercial products, such as those from Ambion, Inc., can also protect samples from degradation prior to nucleic acid purification. Samples can also be freeze-dried, as discussed below in the question, *How Can You Maximize the Storage Life of Purified DNA?*.

3. Thorough, rapid homogenization is crucial. Review the literature to determine if your sample requires any special physical or mechanical means to generate the lysate.

4. Load the appropriate amount of sample. Nothing will impair the quality and yield of a purification strategy more than overloading the system. Too much sample can cause an increase in the viscosity of the DNA preparation and lead to shearing of genomic DNA. If you do not know the exact amount of starting material, use 60 to 70% of your estimate.

### **How Can You Maximize the Storage Life of Purified DNA?**

The integrity of purified DNA in solution could be compromised by nuclease, pH below 6.0 and above 9.0, heavy metals, UV light, and oxidation by free radicals. EDTA is often added to chelate divalent cations required for nuclease activity and to prevent heavy metal oxidative damage. Tris-based buffers will provide a safe pH of 7 to 8 and will not generate free radicals, as can occur with PBS (Miller, Thomas, and Frazier, 1991; Muller and Janz, 1993). Free-radical oxidation seems to be a key player in breakdown and ethanol is the best means to control this process (Evans et al., 2000).

Low temperatures are also important for long-term stability. Storage at 4°C is only recommended for short periods (days) (Krajden et al., 1999). Even though some studies have shown that storage under ethanol is safe even at elevated temperatures (Sharova, 1977), better stability is obtained at -80°C. Storage at -20°C can lead to degradation, but this breakdown is prevented by the addition of carrier DNA. RNA stored in serum has also been shown to degrade at -20°C (Halfon et al., 1996).

Another approach for intermediate storage is freeze drying DNA-containing samples intact (Takahashi et al., 1995). The DNA within freeze-dried tissue was stable for 6 months, but RNA began degrading after 10 weeks of storage. The control of moisture and temperature had a significant effect on shelf life of samples. The long term stability of DNA-containing samples is still being investigated (Visvikis, Schlenck, and Maurice, 1998), but some companies offer specialized solutions (e.g., RNA Later™ from Ambion, Inc.) allowing storage at room temperature.

## **ISOLATING DNA FROM CELLS AND TISSUES**

### **What Are the Fundamental Steps of DNA Purification?**

The fundamental processes of DNA purification from cells and tissues are sample lysis and the segregation of the nucleic acid away from contaminants. While DNA is more or less universal to all species, the contaminants and their relative amounts will differ

considerably. The composition of fat cells differs significantly from muscle cells. Plants have to sustain high pressure, contain chloroplasts packed with chromophores, and often have a very rigid outer cell wall. Bacteria contain lipopolysaccharides that can interfere with purification and cause toxicity problems when present in downstream applications. Fibrous tissues such as heart and skeletal muscle are tough to homogenize. These variations have to be taken into consideration when developing or selecting a lysis method.

### *Lysis*

Detergents are used to solubilize the cell membranes. Popular choices are SDS, Triton X-100, and CTAB(hexadecyltrimethyl ammonium bromide). CTAB can precipitate genomic DNA, and it is also popular because of its ability to remove polysaccharides from bacterial and plant preparations (Ausubel et al., 1998).

Enzymes attacking cell surface components and/or components of the cytosol are often added to detergent-based lysis buffers. Lysozyme digests cell wall components of gram-positive bacteria. Zymolase, and murienase aid in protoplast production from yeast cells. Proteinase K cleaves glycoproteins and inactivates (to some extent) RNase/DNase in 0.5 to 1% SDS solutions. Heat is also applied to enhance lysis. Denaturants such as urea, guanidinium salts, and other chaotropes are applied to lyse cells and inactivate enzymes, but extended use beyond what is recommended in a procedure can lead to a reduction in quality and yield.

Sonication, grinding in liquid nitrogen, shredding devices such as rigid spheres or beads, and mechanical stress such as filtration have been used to lyse difficult samples prior to or in conjunction with lysis solutions. Disruption methods are discussed at [http://www.thescientist.com/yr1998/nov/profile2\\_981109.html](http://www.thescientist.com/yr1998/nov/profile2_981109.html).

### *Segregation of DNA from Contaminants*

The separation of nucleic acid from contaminants are discussed below within the question, *What Are The Strengths and Limitations of Contemporary Purification Methods?*

### *DNA Precipitation*

To concentrate nucleic acids for resuspension in a more suitable buffer, solvents such as ethanol (75–80%) or isopropanol (final concentration of 40–50%) are commonly used in the presence of salt to precipitate nucleic acids (Sambrook, Fritsch, and Maniatis,



1989; Ausubel et al., 1998). If volume is not an issue, ethanol is preferred because less salt will coprecipitate and the pellet is more easily dried. Polyethylene glycol (PEG) selectively precipitates high molecular weight DNA, but it is also more difficult to dry and can interfere with downstream applications (Hillen, Klein, and Wells, 1981). Trichloroacetic acid (TCA) precipitates even low MW polymers down to (5kDa) (<http://biotech-server.biotech.ubc.ca/biotech/bisc437/lecture/e-na-isoln/na-isoln3.html>), but nucleic acids cannot be recovered in a functional form after precipitation.

Salt is essential for DNA precipitation because its cations counteract the repulsion caused by the negative charges of the phosphate backbone. Ammonium acetate is useful because it is volatile and easily removed, and at high concentration it selectively precipitates high molecular weight molecules. Lithium chloride is often used for RNA because  $\text{Li}^+$  does not precipitate double-stranded DNA, proteins, or carbohydrates, although the single-stranded nucleic acids must be above 300 nucleotides. To efficiently precipitate nucleic acids, incubation at low temperatures (preferably  $\leq -20^\circ\text{C}$ ) for at least 10 minutes is required, followed by centrifugation at  $12,000 \times g$  for at least five minutes. Temperature and time are crucial for nucleic acids at low concentrations, but above 0.25 mg/ml, precipitation may be carried out at room temperature. Additional washing steps with 70% ethanol will remove residual salt from pelleted DNA. Pellets are dried in a speed vac or on the bench and are resuspended in water or TE (10 mM Tris, 1 mM EDTA). Do not attempt to precipitate nucleic acids below a concentration of 20 ng/ml unless carrier such as RNA, DNA, or a high molecular weight co-precipitant like glycogen is added. In the range from 20 ng/ml to 10  $\mu\text{g/ml}$ , either add carrier or extend precipitation time, and add more ethanol. Polyethylene glycol (PEG) precipitation is even more concentration dependent and will only work at DNA concentrations above 10  $\mu\text{g/ml}$  (Lis and Schleif, 1975). Pellets will dissolve better in low-salt buffers (water or TE) and at concentrations below 1 mg/ml. Gentle heating can also help to redissolve nucleic acids

## **What Are the Strengths and Limitations of Contemporary Purification Methods?**

### *Salting out and DNA Precipitation*

#### Mechanism

Some of the first DNA isolation methods were based on the use of chaotropes and cosmotropes to separate cellular components

based on solubility differences (Harrison, 1971; Lang, 1969). A chaotrope increases the solubility of molecules (“salting-in”) by changing the structure of water, and as the name suggests, the driving force is an increase in entropy. A cosmotrope is a structure-maker; it will decrease the solubility of a molecule (“salting-out”). Guanidium salts are common chaotropes applied in DNA purification. Guanidinium isothiocyanate is the most potent because both cation and anion components are chaotropic. Typical lyotropes used for salting out proteins are ammonium and potassium sulfate or acetate. An all solution based nucleic acid purification can be performed by differentially precipitating contaminants and nucleic acids.

Cells are lysed with a gentle enzyme- or detergent-based buffer (often SDS/proteinase K). A cosmotrope such as potassium acetate is added to salt out protein, SDS, and lipids but not the bulk of nucleic acids. The white precipitate is then removed by centrifugation. The remaining nucleic acid solution is too dilute and in a buffer incompatible with most downstream applications, so the DNA is next precipitated as described above.

#### Features

Protocols and commercial products differ mainly in lysis buffer composition. Yields are generally good, provided that sample lysis was complete and DNA precipitation was thorough. These procedures apply little mechanical stress, so shearing is generally not a problem.

#### Limitations

If phenolic contaminants (i.e., from plants) are a problem, adding 1% polyvinylpyrrolidone to your extraction buffer can absorb them (John, 1992; Pich and Schubert, 1993; Kim et al., 1997). Alternatively, add a CTAB precipitation step to remove polysaccharides (Ausubel et al., 1998).

### *Extraction with Organic Solvents, Chaotropes, and DNA Precipitation*

#### Mechanism

Chaotropic guanidinium salts lyse cells and denature proteins, and reducing agents ( $\beta$ -mercaptoethanol, dithiothreitol) prevent oxidative damage of nucleic acids. Phenol, which solubilizes and extracts proteins and lipids to the organic phase, sequestering them away from nucleic acids, can be added directly to the lysis buffer, or a phenol step could be included after lysis with either

GTC- or SDS-based buffers as above. GTC/phenol buffers often require vortexing or vigorous mixing.

The affinity of nucleic acids for this two-phase extraction system is pH dependent. Acidic phenol is applied in RNA extractions because DNA is more soluble in acidic phenol; smaller DNA molecules (<50kb) will be found in the organic phase and larger DNA molecules (>50kb) in the interphase. When purifying RNA via this procedure, it is essential to shear the DNA to ensure a light interphase.

Phenol titrated to a pH of 8 is used to separate DNA from proteins and lipids, since DNA is insoluble in basic phenol. Whether protocols call for a GTC/phenol, a GTC, or an SDS based step followed by phenol, it is best to follow a phenol extraction with chloroform in order to extract residual phenol from the aqueous phase. Phenol is highly soluble in chloroform, and chloroform is not water soluble. Remaining lipids may also be removed by this step. Phenol extractions are followed by nucleic acid precipitation steps as described above.

#### Features

Though caustic and toxic, this strategy still has wide use because yield, purity, and speed are good, and convenient for working with small numbers of samples.

#### Limitations

If lysis is incomplete, the interphase between organic and aqueous layers becomes very heavy and difficult to manipulate, and may trap DNA. Phenol is not completely insoluble in water, so if chloroform steps are skipped, residual phenol can remain and interfere with downstream applications. High salt concentrations can also lead to phase inversion, where the aqueous phase is no longer on top (problematic if colorless phenol is used). Diluting the aqueous phase and increasing the amount of phenol will correct this inversion. When working with GTC/phenol-based extraction buffers, cross-contamination of RNA with DNA, and vice versa, is frequent.

### *Glass Milk/Silica Resin-Based Strategies*

#### Mechanism

Nucleic acids bind to glass milk and silica resin under denaturing conditions in the presence of salts (Vogelstein and Gillespie, 1979). Recent findings indicate that binding of some nucleic

acids might even be feasible under nondenaturing conditions (Neudecker and Grimm, 2000). The strong, hydrophobic interaction created in the presence of chaotropic substances can be easily disrupted by removal of salt. The adsorption is followed by wash steps, usually with salt/ethanol which will not interfere with the strong binding of nucleic acids but will wash away remaining impurities and excess chaotrope. Depending on the protocol, this can be followed by a low salt/ethanol wash step that can lead to a reduction in yield. Finally nucleic acids are eluted from the glass in a salt or TE buffer. Nucleic acids are then ready for use.

Most methods create a denaturing adsorption environment by using guanidium salts for one-step lysis and binding. The strength of the binding depends on the cation used to shield the negative charges of the phosphate backbone and the pH (Romanowski et al., 1991). Slightly acidic pH and divalent cations, preferably magnesium, seem to work best.

Differences between glass milk, silica resin, and powdered glass consist mainly in capacity and adsorption strength, a function of impurities present in the binding resins. Diatomaceous earths seem to have an especially high binding capacity (<http://www.nwfsc.noaa.gov/protocols/dna-prep.html>). Pure silica oxide has the lowest affinity to nucleic acids (Boom et al., 1990), but this can improve recovery even though initial binding capacity is lower.

Glass milk is silica presuspended in chaotropic buffer, whereas the silica resin is a solid, predispensed matrix usually found in spin or vacuum flow-through format. Glass milk gives more flexibility for scale of prep, predispensed resin is more convenient for high-throughput applications. Glass milk or silica-based kits are available from numerous vendors, and even though the basic principle is the same, there can be significant differences in efficiency, purity, and yield.

### Features

DNA purification based on hydrophobic adsorption to glass or silica is fast, simple, straightforward, and scalable. No additional time-consuming and yield-reducing precipitation steps are required. Depending on binding and wash buffer composition, very good yield and purity values are obtained. This purification approach can also allow restriction digestion/ligation reactions directly on the glass surface, improving transformation efficiency of complex ligation mixtures (Maitra and Thakur, 1994).

### Limitations

One of the dangers of silica-based strategies is underloading the sample. Even though yields are good, there is always sample loss due to some remaining material on the resin or filter. The smaller the DNA fragment, the tighter is the interaction. Oligonucleotide primers are actually removed because binding to the glass becomes virtually irreversible. Underloading can become a critical issue when working with small samples and large volumes of glass milk or silica filter.

Some of the older methods utilized unstable buffer components, such as NaI, that tended to oxidize over time, leading to very poor recoveries. Some procedures required the addition of reagents to produce functional wash or elution buffers. If the concentrations were incorrect, or if volatile reagents (i.e., ethanol) were added and the buffers stored long term, these buffers lost their effectiveness. Incomplete sample lysis can be problematic because intact cells may also bind to silica and lyse under low- or no-salt elution conditions, leading to degradation of nucleic acids. Incomplete ethanol removal after wash steps will cause the problems described earlier for ethanol precipitation (discussed below under the question *What Are The Fundamental Steps Of DNA Purification?*). Ethanol must be completely removed from the samples after wash steps to avoid problems such as diffusion out of agarose gel wells (“unloadable” DNA/RNA) or undigestable DNA. Overdrying will lead to irreversible binding of nucleic acids to the resin severely impairing yields.

### *Anion Exchange (AIX) Based Strategies*

#### Mechanism

Nucleic acids are very large anions with a charge of  $-1/\text{base}$  and  $-2/\text{bp}$ ; hence they will bind to positively charged purification resins (commonly referred to as anion exchangers). After washes in low-salt buffers, the DNA is eluted in a high-salt buffer. AIX strategies are applied to purify genomic and plasmid DNA.

Logic might suggest that the greater the strength of the anion exchanger, the more DNA it would bind (and more tightly), which would make for superior DNA purification. In practice, however, if an anion exchanger is too strong, most DNA is never recovered. This is especially problematic when working with small samples and with spun column formats. Forcing liquid through porous chromatography resins via centrifugation does not allow for even flow rates, hence resolution is poor. For this reason some spun column plasmid purification procedures advise the recovery of

only a portion of the potential total material to avoid contamination by genomic DNA. Procedures where the buffer flows through columns packed with AIX resins under the force of gravity (as in standard column chromatography) can overcome this problem, but are slower. Gravity flow-based columns can clog if lysis is incomplete or if removal of protein or lipid is incomplete. Resolution is very much flow rate dependent, and tight control of linear flow rates on HPLC or FPLC™ systems are superior to gravity flow and/or spun column formats when it comes to resolution and scale-up.

#### Features

These methods can produce very pure DNA, but the yields in small-scale applications tend to be low, especially in spun column formats.

#### Limitations

Not the most robust method, and recoveries tend to be lower, and the final elution step of AIX protocols involves high-salt buffers. The 0.7 to 2M sodium chloride eluate needs to be desalted, usually by a precipitation step, which decreases recovery and increases the overall procedure time. The binding capacities tend to be low (0.25–2mg/ml of resin), increase with pH, and decrease with increasing size of the DNA. The amount of RNA present in the sample will also affect binding capacity because RNA will compete with DNA for binding.

### *Hydroxyapatite (HA) Based Strategies*

#### Mechanism

Nucleic acids bind to crystalline calcium phosphate through the interaction of calcium ions on the hydroxyapatite and the phosphate groups of the nucleic acids. An increase in competing free phosphate ions from 0.12 to 0.4M will elute nucleic acids, with single-stranded nucleic acids eluting before double-stranded DNA. The entire experiment needs to be run at 60°C for thermal elution (Martinson and Wagenaar, 1974) or in the presence of formamide at room temperature (Goodman et al., 1973).

Sodium phosphate buffers are most commonly used; the phosphate salt affects the selectivity of the resin (Martinson and Wagenaar, 1974). Nucleic acids may also be eluted by increasing the temperature until nucleic acid strands melt and elute from the column.

### Features

Excellent separation of single-stranded from double-stranded DNA molecules.

### Limitations

The quality and performance of hydroxyapatite can vary from batch to batch and between manufacturers. Thermal elution procedures require reliable temperature control, but fluctuations occur because of lack of heat-regulated chromatography equipment. These elevated temperatures can also produce bubbles in the buffer that can interfere with the separation. Hydroxyapatite has poor mechanical stability. Hydroxyapatite procedures often employ high-salt buffers and lead to sample dilution, requiring an additional precipitation step.

For these reasons hydroxyapatite is not extensively referenced. It is mostly limited to subtractive cDNA cloning (Ausubel et al., 1998), removal of single-stranded molecules, and DNA re-association analysis (Britten, Graham, and Newfeld, 1974).

## **What Are the Steps of Plasmid Purification?**

### *Alkaline Lysis and Boiling Strategies*

#### Mechanism (Small Scale)

Plasmid purification holds a special challenge because the target DNA must be purified from DNA contaminants. Isolation strategies take advantage of the physical differences between linear, closed, and supercoiled DNA. Alkaline lysis (Birnboim and Doly, 1979), boiling, and all other denaturing methods exploit the fact that closed DNA will renature quickly upon cooling or neutralizing, while the long genomic DNA molecules will not renature and remain “tangled” with proteins, SDS, and lipids, which are salted out. Whether boiling or alkaline pH is the denaturing step, the renaturing step is usually performed in the cold to enhance precipitation or salting-out of protein and contaminant nucleic acids.

Buffer 1 of an alkaline lysis procedure contains glucose to buffer the effects of sodium hydroxide added in step 2, and lysozyme, to aid cellular breakdown which prevents plasmid from becoming trapped in cellular debris. Buffer 2 contains SDS and NaOH. SDS denatures proteins and NaOH denatures DNA, both plasmid and genomic, and proteins, and partially breaks down RNA. Buffer 3 contains an acidic potassium acetate solution that will salt out proteins by complexing SDS with potassium and precipitating out a mix of SDS, K<sup>+</sup>, proteins, and denatured genomic

DNA. Supercoiled plasmids and RNA molecules will remain in solution.

Another method lyses cells by a combination of enzymatic breakdown, detergent solubilization, and heat (Holmes and Quigley, 1981). The lysis buffer usually contains lysozyme, STE, and Triton X-100 or CTAB. Bacterial chromosomal DNA remains attached to the membrane and precipitates out. Again, the aqueous supernatant generated by this method contains plasmid and RNA.

Polyethylene glycol (PEG) has been used to separate DNA molecules by size, based on its size-specific binding to DNA fragments (Humphreys, Willshaw, and Anderson, 1975; Hillen, Klein, and Wells, 1981). A 6.5% PEG solution can be used to precipitate genomic DNA selectively from cleared bacterial lysates. Trace amounts of PEG may be removed by a chloroform extraction.

Isolation of plasmid DNA by cesium chloride centrifugation in the presence of ethidium bromide (EtBr) is especially useful for large-scale DNA preparations. The interaction of EtBr with DNA decreases the density of the nucleic acid; because of its supercoiled conformation and smaller size, plasmid incorporates less EtBr than genomic DNA, enhancing separation on a density gradient.

Chromatographic methods such as anion exchange and gel filtration may also be used to purify plasmids after lysis. For chromatography, RNA removal prior to separation is essential because the RNA will interfere with and contaminate the separation process. RNase A treatments (Feliciello and Chinali, 1993), RNA-specific precipitation (Mukhopadhyay and Mandal, 1983; Kondo et al., 1991), tangential flow filtration (Kahn et al., 2000), and nitrocellulose filter binding (Levy et al., 2000a, 2000b) have been employed to desalt, concentrate, and generally prepare samples for column purification.

### Limitations

The efficiency of plasmid purification will vary with the host cell strain due to differences in polysaccharide content and endonuclease—End A+ strains such as HB101 (Ausubel et al., 1998). Recombination impaired hosts are often selected when producing plasmids prone to deletion and rearrangement of cloned inserts (Summers and Sherratt, 1984; Biek and Cohen, 1986). The University of Birmingham's Web site gives useful links to research strain genotypes and characteristics at <http://web.bham.ac.uk/bcm4ght6/res.html>, as does the *E. coli* Genetic Stock Center at Yale University (<http://cgsc.biology.yale.edu>).



Whenever nucleic acids are denatured, there is a risk of irreversible denaturation. Never increase the denaturation time beyond what is recommended, and ensure that pH values are accurate for neutralization. Prolonged high pH or heat exposure may lead to more contamination with genomic DNA (Liou et al., 1999) and nicked, open, and irreversibly denatured plasmid. The pH of solution 3 of an alkaline lysis procedure needs to be pH 5.5 to precipitate out SDS/protein/genomic DNA. Effects of changing critical parameters have been studied in detail (Kieser, 1984). These protocols have been modified to purify cosmids, but larger DNA molecules will not renature as well as small plasmids. Most methods work well for plasmids up to 10 kb; above 10 kb, denaturation has to be milder (Hogrefe and Friedrich, 1984; Azad, Coote, and Parton, 1992; Sinnett, Richer, and Baccichet, 1998).

The yield of low copy number plasmids can be improved dramatically by adding chloramphenicol (Norgard, Emigholz, and Monahan, 1979) or spectinomycin (300  $\mu$ g/ml; Amersham Pharmacia Biotech, unpublished observations), which prevent replication of chromosomal but not plasmid DNA. However, extended exposure to such agents have also been shown to damage DNA in vitro (Skolimowski, Knight, and Edwards, 1983).

### Resources

Plasmid purification methodology could fill an entire book of its own. Traditional chromatography has been applied to isolate large- and small-scale preparations of plasmid from a variety of hosts. Techniques include gel filtration, anion exchange, hydrophobic interaction chromatography, single-strand affinity matrix (Pham, Chillapagari, and Suarez, 1996; Yashima et al., 1993a, b), triple helix resin, silica resin, and hydroxyapatite in a column as well as microtiter plate format. Plasmid purification procedures are reviewed in O'Kennedy et al. (2000), Neudecker and Grimm (2000), Monteiro et al. (1999), Ferreira et al., 2000. Ferreira et al. (1999), Ferreira et al. (1998), Huber (1998), Lyddiatt and O'Sullivan (1998), and Levy et al. (2000a).

### *CsCl Purification*

#### Mechanism

The separation of DNA from contaminants based on density differences (isopycnic centrifugation) in CsCl gradients remains an effective if slow method. High *g* forces cause the migration of dense Cs<sup>+</sup> ions to the bottom of the tube until centripetal force and force of diffusion have reached an equilibrium.

Within a CsCl gradient, polysaccharides will assume a random coil secondary structure, DNA a double-stranded intermediate density conformation, and RNA, because of its extensive secondary structure, will have the highest density. Dyes that bind to nucleic acids and alter their density have been applied to enhance their separation from contaminants. The binding of EtBr decreases the apparent density of DNA. Supercoiled DNA binds less EtBr than linear DNA, enhancing their separation based on density differences. CsCl centrifugation is most commonly applied to purify plasmids and cosmids in combination with EtBr. Ausubel et al. (1998) also provides protocols for the isolation of genomic DNA from plants and bacteria.

#### Features

Cesium gradient formation requires long periods (at least overnight) of ultracentrifugation and are caustic, yet remain popular because they produce high yield and purity and are more easily scaled up.

#### Limitations

GC content of DNA correlates directly to its density. Equilibrium density of DNA can be calculated as  $1.66 + 0.098 \times \%GC$  (Sambrook, Fritsch, and Maniatis, 1989). The density of very GC-rich DNA can be sufficiently high as to cause it to migrate immediately adjacent to RNA in a CsCl gradient. If too much sample is loaded onto a gradient, or if mistakes were made during preparation of the gradient, separation will be incomplete or ineffective.

#### *Affinity Techniques*

Triple helix resins have been used to purify plasmids and cosmids (Wils et al., 1997). This approach takes advantage of the adoption of a triple rather than a double helix conformation under the proper pH, salt, and temperature conditions. Triple helix affinity resins are generated by insertion of a suitable homopurine sequence into the plasmid DNA and crosslinking the complement to a chromatographic resin of choice. The triple helix interaction is only stable at mild acidic pH; it dissociates under alkaline conditions. The interaction at mildly acid pH is very strong (Radhakrishnan and Patel, 1993). This strong affinity allows for extensive washing that can improve the removal of genomic DNA, RNA, and endotoxin during large-scale DNA preparations.

A radically different approach applies covalent affinity chromatography to trap contaminants. Some of the examples include

a chemically modified silica resin that irreversibly binds protein via an imide bond (Ernst-Cabrera and Wilchek, 1986), and a modified silica resin that covalently binds to polysaccharides via a cyclic boric acid ester, trapping proteins in the process. This latter reaction was initially applied to purify tRNA (McCutchan, Gilham, and Soll, 1975); it is described in greater detail by O'Neill et al. (1996). Some commercial products use salts to generate an irreversible protein precipitate that forms a physical barrier between the aqueous nucleic acid and the solid protein phase. Affinity-based technologies are also described at <http://www.polyprobe.com/about.htm> and at <http://www.edgebio.com>.

#### Features

Affinity techniques can produce excellent yields. Impressive purity is achieved if the system is not overloaded; if need be, the affinity steps can be repeated to further enhance purity. These methods are especially recommended when sample is precious and limited or purity requirements are very high.

#### Limitations

Cost, which may be minimized by reuse of resin. However cleaning of resin and its validation may be problematic.

## **WHAT ARE THE OPTIONS FOR PURIFICATION AFTER IN VITRO REACTIONS?**

### **Spun Column Chromatography through Gel Filtration Resins**

#### *Mechanism*

As in standard, column-based gel filtration (size exclusion) chromatography, a liquid phase containing sample and contaminant passes through a resin. The smaller molecules (contaminant) enter into the resin's pores, while the larger molecules (desired product) will pass through without being retained. Properly applied, this procedure can accomplish quick buffer exchange, desalting, removal of unincorporated nucleotides, and the elimination of primers from PCR reactions (gel filtration spin columns will *not* remove enzyme from a reaction; this requires organic extraction) to name a few applications.

#### *Features and Limitations*

These procedures are fast, efficient, and reproducible when the correct resins and centrifugation conditions are applied to the

appropriate samples. Viscous solutions are not compatible with this technique.

One should not approach spun column, size exclusion chromatography with a care-free attitude. The exclusion limits based on standard chromatography should not be automatically applied to spun columns. Spinning makes such standard chromatography data obsolete. Before you apply a resin or a commercial spun column in an application, verify that the product has been successfully used in your particular application. Just because a resin has a pore size that can exclude a 30 nucleotide long oligo isn't a guarantee that a column with this resin will remove all or even most of the primer from a PCR reaction.

Manufacturers will optimize the columns and/or the procedures to accomplish a stated task. The presence of salt (100–150mM NaCl) improves the yield of radiolabeled probes from one type of spun column, but the presence of Tris can interfere with the preparation of templates for automated sequencing (Amersham Pharmacia Biotech, unpublished observations, and *Nucleic Acid Purification Guide*, 1996). Too much *g* force, and the contaminants can elute with the desired product; too little *g* force, and the desired product is not eluted. If the volume you're eluting off the spun column is much greater or less than the volume you've loaded, the applied *g* force is no longer correct.

If you plan to create a spun column from scratch, consider the following:

- Sample volumes should be kept low with respect to the volume of resin, usually below a tenth to a twentieth of the column volume to allow for good resolution.
- Gel filtration resin will not resolve components efficiently (purity >90%) unless the largest contaminant is at least 20 times smaller than the smallest molecule to be purified.
- Desalting, where the size difference between ions and bio-molecule is >>1:20, works well even at high flow rates.

## **Filter Cartridges**

### *Mechanism*

Filtration under the influence of vacuum suction or centrifugation operates under principles similar to gel filtration. Semipermeable membranes allow passage of small molecules such as salts, sugars, and so forth, but larger molecules such as DNA are retained. Since the retentate rather than an eluate is collected, samples will be concentrated. Ultrafiltration and microfiltration are reviewed by Munir (1998) and Schratter et al., (1993).

### *Features and Limitations*

Filtration procedures are fast and reproducible provided that the proper *g* force or vacuum are applied. Membranes can clog from debris when large molecules accumulate at the membrane surface (but don't pass through), forming a molecule-solute gel layer that prevents efficient removal of remaining contaminants. As with gel filtration spun columns, filtration will not remove enzymes from reaction mixes unless the enzyme is small enough to pass through the membrane, which rarely is the case.

## **Silica Resin-Based Strategies**

### *Mechanism*

The approach is essentially identical to that described for silica resins used to purify DNA from cells and tissue, as described above.

### *Features and Limitations*

Advantages and pitfalls are basically the same. Recoveries from solutions are between 50 to 95% and from agarose gels, 40 to 80%. Fragments smaller than 100 bp or larger than 10 kb (gel), or 50 kb (solution), are problematic. Small fragments may not elute unless a special formulation of glass milk is used (e.g., Glass Fog™ by 5'-3' Eppendorf), and large fragments often shear and give poor yield. Depending on the capture buffer formulation, RNA and single-stranded molecules may or may not bind.

When using silica resins to bind nonradioactively labeled probes, investigate the stability of the label in the presence of chaotrope used for the capture and washing steps. Chaotropes create an environment harsh enough to attack contaminants such as proteins and polysaccharides, so it would be prudent to assume that any protein submitted to such an environment will lose its function. Nucleic acids covalently tagged with horseradish peroxidase or alkaline phosphatase are less likely to remain active after exposure to harsh denaturants. The stability of the linker connecting the reporter molecule to the DNA should also be considered prior to use.

Also consider the effect of reporter molecules/labels on the ability of DNA to bind to the resin. Nucleic acids that elute well in the unlabeled state may become so tightly bound to the resin by virtue of their label that they become virtually "sorbed out" and hence are unrecoverable. This is a notable concern when the reporter molecule is hydrophobic.

## Isolation from Electrophoresis Gels

This subject is also addressed in Table 8.4 of Chapter 8, “Electrophoresis.” Purification through an electrophoresis gel (referred to hereon as *gel purification*) is the only choice if the objective is to simultaneously determine the fragment size and remove contaminants. It could be argued that gel purification is really a two-step process. The first step is filtration through the gel and separation according to size. The second step is required to remove impurities introduced by the electrophoresis step (i.e., agarose, acrylamide, and salts). There are several strategies to isolate DNA away from these impurities, as summarized in Table 7.1 and discussed in detail below.

All these procedures are sensitive to the size and mass of the amount of gel segment being treated. The DNA should appear on the gel as tight bands, so in the case of agarose gels, combs must be inserted straight into the gel. When isolating fragments for cloning or sequencing, minimize exposure to UV light; visualize the bands at 340nm. Any materials coming in contact with the gel slice should be nuclease free. Crush or dice up the gel to speed up your extraction method.

### *Polyacrylamide Gels*

#### Crush and Soak

With time, nucleic acids diffuse out of PAGE gels, but recovery is poor. The larger the fragment size, the longer is the elution time required for 50% recovery. Elevated temperatures (37°C) accelerate the process. A variation of the crush and soak procedure is available at [http://www.ambion.com/techlib/tb/tb\\_171.html](http://www.ambion.com/techlib/tb/tb_171.html). A procedure for RNA elution is provided at <http://grimwade.biochem.unimelb.edu.au/bfjones/gen7/m7a4.htm>.

#### Electroelution

Depending on the instrumentation, electroelution can elute DNA into a buffer-filled well, into a dialysis bag, or onto a DEAE cellulose paper strip inserted into the gel above and below the band of interest. Inconsistent performance and occasionally difficult manipulations make this approach less popular.

#### Specialized Acrylamide Crosslinkers

These are discussed in Chapter 8, “Electrophoresis.”

Table 7.1 Comparison of Nucleic Acid Purification Methods from Gel and/or Solution

| Method                                                                                  | Used for                                                               | Yield                                                 | Speed                                                                                                             | Benefits                                                                                                                                                                                            | Limitations                                                                                                                                                               |
|-----------------------------------------------------------------------------------------|------------------------------------------------------------------------|-------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| I.m.p. agarose with or without agarase, or phenol" (Ausubel et al., 1998, Hengen, 1994) | DNA fragments and/or plasmids                                          | Up to 70%; typically 50%                              | From 0.5 to 2h depending on downstream purification method chosen                                                 | Agarase especially useful for large fragments or cosmids, since treatment is very gentle; some applications may allow treatment directly in melted gel slice (e.g., ligation, labeling with Klenow) | Requires an additional purification step; carrier often required for precipitation because solutions are dilute; extraction with phenol is caustic, especially hot phenol |
| "Freeze and squeeze" (Benson and Spencer, 1984, Ausubel et al., 1998)                   | DNA, RNA fragments                                                     | 40–60% (for fragments up to 5 kb, above that, lower)  | Slow: freeze for at least 15' or up to 2 h, then follow with precipitation                                        | Very gentle; good for larger molecules; very inexpensive                                                                                                                                            | Low yield                                                                                                                                                                 |
| "Crush and soak" (for acrylamide gels) Sambrook, Fritsch, and Maniatis, 1989            | Mostly RNA, but works for any nucleic acid                             | 40–70% depending on elution time, concentration, etc. | 2–4h depending on fragment size                                                                                   | Allows high sample loads; best for reactions generating larger quantities of probe (in vitro transcription) to compensate for low recoveries                                                        | Significant chance of contamination when working with radioactivity; poor recovery                                                                                        |
| Gel filtration, desalting Ausubel et al., 1998; Sambrook, Fritsch, and Maniatis, 1989   | DNA and RNA; fragment size must be well above exclusion limit of resin | >90% for fragments above exclusion limit              | 3–15 min, depending on column format (gravity flow vs. spun column); primer removal protocols might require 30min | Fast, with high purity and yield                                                                                                                                                                    | Often leads to dilution of sample; only removes small contaminants, difficult to monitor separation of noncontaminants without radioactivity                              |
| Glass milk/Na I (Ausubel et al., 1998; Hengen, 1994)                                    | Usually DNA fragments and plasmids from agarose gel or solution        | 50–75% from solution, 40–70% from gel                 | 0.25–1.5h                                                                                                         | Fast, versatile; removes most major contaminants (proteins, primers, salts); efficient one-step purification                                                                                        | Yield: Na I stability; shearing                                                                                                                                           |

|                                                                                                                                                                                                                     |                                                                                                          |                                                                                                   |                                                                                                |                                                                                                                                |                                                                                                                                                                                  |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Silica/guanidinium salts (Ausubel et al., 1998; Gribovanov et al., 1996; Boom et al., 1990; Vogelstein and Gillespie, 1979)                                                                                         | Usually DNA fragments and plasmids from agarose gel or solution                                          | 80–90% from solution, up to 80% from gel                                                          | 5 min from solution; up to 1 h from gel                                                        | Fast, versatile; removes most major contaminants (proteins, primers, salts); efficient one-step purification                   | Shearing; yields very much resin/protocol-dependent                                                                                                                              |
| Filter cartridges in combination with freezing or without freezing (Leonard et al., 1998; Blattner et al. 1994; Li and Ownby, 1993; Schwarz and Whitton, 1992 )                                                     | Concentration and desalting of DNA/RNA samples; desalting of freeze-squeeze eluted agarose gel slices    | Up to 95% depending on fractionation range of membrane and nonspecific interaction with membrane  | Often 2–5 min; depends on required concentration and salt tolerance of downstream applications | Fast; simultaneous desalting and concentration possible                                                                        | Molecular size cutoff is not always well defined; not recommended for primer removal unless size cutoff well above primer size; will not remove large contaminants like proteins |
| Ethanol or isopropanol precipitation (Sambrook, Fritsch, and Maniatis, 1989; Ausubel et al., 1998)                                                                                                                  | Any nucleic acid as long as concentration is >10 µg/ml and at least 0.1 M monovalent cations are present | Up to 95% depending on protocol                                                                   | 20 min–overnight depending on sample concentration                                             | Easy to monitor (visible pellet); noncaustic, robust, high yields, versatile in combination with different precipitation salts | More time-consuming; difficult for multiple samples, pellet may be lost; may not remove protein contaminants                                                                     |
| Electroelution (Ausubel et al., 1998; Bostian, Lee, and Halvorson, 1979, Dretzen et al., 1981, Girvitz et al., 1980; Henrich, Lubitz, and Fuchs, 1982; Smith, 1980; Strongin et al., 1977; Tabak and Flavell, 1978) | Mostly DNA fragments from gel; elution onto DEAE membrane does not work well for fragments >2 kb         | Up to 90% for fragments <1 kb, very small fragments between 50–60%, large fragments as low as 20% | 2–4 h; or 1–3 h for DEAE elution                                                               | Few reagents required; not caustic/toxic; yields for fragments up to 1 kb are quite high                                       | More difficult to monitor; only for fragments from 0.05–20 kb; need to be combined with a second method                                                                          |

Source: Data in table aside from references also based on average values found in catalogs and online of the following manufacturers: Ambion, Amersham Pharmacia Biotech, Amresco, Bio101, BioRad, Biotronics, BioTeex, Bioventures, Boehringer Mannheim/Roche, Clontech, CPG, Dynal, Edge Biosystems, Epicentre, FMC, Genhunter, Genosys, Gentra Systems, GIBCO Life Technologies, Invitrogen, Ligochem, 5'3' (Eppendorf), Macromolecular Resource Center, Maxam Biotech, MBI Fermentas PerSeptive (now Life Technologies), Nucleon, Promega, Qiagen, Schleicher & Schuell, Sigma, Stratagene, USB, Worthington. For additional data, see DeFrancesco (1999), who provides a fragment purification products table and a comparison of size, agarose limitations, buffer compatibility, time requirements, yield, capacity, and volume for isolation of DNA from agarose gels.

<sup>a</sup> If hot phenol is used, avoid phenol chloroform which can severely impair yields (Ausubel et al., 1998).



## Agarose

Detailed procedures regarding the methodology discussed below are available at <http://www.bioproducts.com/technical/dnarecovery.shtml#elution>.

### Freeze and Squeeze

Comparable to crush and soak procedures for polyacrylamide gels, this method is easy and straightforward, but it suffers from poor yields.

### Silica-Based Methods

Silica or glass milk strategies are fast and efficient because the same buffer can be used for dissolving the gel and capturing the nucleic acid. Problems may arise when agarose concentrations are very high (larger volumes of buffers are required, reducing DNA concentration), nucleic acid concentration is very low (recovery is poor), fragment size is too small or large (irreversible binding and shearing, respectively), or if agarose dissolution is incomplete. Finally, some silica resins will not bind nucleic acids in the presence of TBE. When in doubt use TAE buffers (Ausubel et al., 1998).

### Low Melting Point Agarose (LMP Agarose)

LMP agarose melts between 50 and 65°C. Some applications tolerate the presence of LMP agarose (Feinberg and Vogelstein, 1984), but for those that don't, DNA can be precipitated directly or isolated by phenol treatment ([http://mycoplasmas.vvm.iastate.edu/lab\\_site/methods/DNA/elutionagarose.html](http://mycoplasmas.vvm.iastate.edu/lab_site/methods/DNA/elutionagarose.html)). Another option is to digest the agarose with agarase. This DNA can either be used directly for some applications or be precipitated to remove small polysaccharides and concentrate the sample. Glass beads are another way to follow up on melting your agarose slice as mentioned above. The negative aspect of LMP agarose is that sample load and resolution power are lower than in standard agarose procedures.

## What Are Your Options for Monitoring the Quality of Your DNA Preparation?

The limitations of assessing purity by  $A_{260}:A_{280}$  ratio are described in Chapter 4 (spectrophotometer section). Nevertheless,  $A_{260}:A_{280}$  ratios are useful as a first estimation of quality. For northern and southern blots, try a dot blot. Success of PCR reactions can be scouted out by amplification of housekeeping genes. If

restriction fragments do not clone well, try purifying a control piece of DNA with the same method and religate.

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